enzyme family. All RNA polymerases are characterized by a relatively unstable initial phase (abortive cycling) and by a significant structural rearrangement associated with the transition to stable elongation. Our previous studies with the single subunit T7 RNA polymerase have established a key role for downstream DNA bubble collapse in the instability of halted initiating complexes. We now extend these studies to the initially transcribing complexes of sigma70-*E. Coli* RNA polymerase transcribing on variants of the T5 N25 promoter. Specifically, we have studied the role of downstream DNA bubble collapse in initially transcribing complexes by designing partially single stranded DNA constructs with the nontemplate absent at the downstream edge of the transcription bubble. As in our earlier studies, the current results show that collapse of the downstream end of the transcription bubble contributes to instability of a halted complex. This increases turnover and hence the amount of abortive transcripts. Transcription to elongation on these constructs show 18-23mer RNA products. We propose that absence of the collapse of the *upstream* end of the transcription bubble impairs proper displacement of the 5'end of RNA into the exit channel, leading to unstable complexes. This parallels our earlier model in the T7 system in which upstream bubble collapse (−4 to +2) facilitates displacement of the RNA from the hybrid (impairment of collapse leads to release of 11-13mer RNAs) enabling the transition to elongation.

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Mechanisms of Eukaryotic Transcription Initiation

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Regulation of gene expression determines cellular phenotype and when disrupted can lead to cancer and other disease states. For many genes regulation occurs via control of transcription initiation. Eukaryotic transcription initiation of mRNA is a complex process wherein a set of general transcription factors and RNA polymerase II (Pol II) assemble into a pre-initiation complex (PIC) at a promoter. The protein factors involved and the physical interactions between them and the promoter have been the subject of many years of research in the field. However, the topological dynamics of the DNA in eukaryotic PICs are not well understood, limiting mechanistic understanding of promoter unwinding, transcription start-site scanning, and promoter escape.

Using magnetic tweezers we have observed ATP-dependent promoter opening by single minimal PICs comprised of TBP, TFIIB, TFIIE, TFIIF and TFIIH and Pol II assembled on the TATA-dependent HIS4 promoter from *Saccharomyces cerevisiae*. Single molecule traces of TFIIH-dependent promoter opening reveal that the size of the transcription bubble is dynamic during initiation, displaying multiple transitions between intermediates with varying extents of open DNA. Furthermore, in contrast to prokaryotic initiation, promoter opening does not appear to be accompanied by significant DNA compaction. These observations place constraints on the promoter opening and transcription start site scanning mechanisms for yeast PICs.

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Use of Fast DNA Footprinting and Real-Time Fluorescence Spectroscopy to Characterize Novel, Biologically Revlevant Transcription Initiation Intermediates for E. Coli RNA Polymerase

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RNA polymerase (RNAP) is a complex molecular motor responsible for catalyzing the synthesis of RNA from DNA in a promoter sequence-dependent manner. Knowledge of the mechanism of transcription initiation is crucial to understanding the regulation of gene expression. However, the RNAP-DNA intermediates on the pathway to open complex formation during transcription initiation are extremely short-lived and thus have been very difficult to characterize using traditional structural methods. Here, we present work focused on developing rapid, solution-based methods that allow us to characterize RNAP-DNA structures in the millisecond time regime. These include: fast DNA footprinting (using hydroxyl radical and permanganate probes) to characterize the interaction of RNAP with the DNA backbone and the extent of thymine base unstacking; stopped-flow FRET experiments to monitor DNA bending in real time; and 2-aminopurine fluorescence experiments to characterize the kinetics of DNA opening. In a related project, we utilize thin layer chromatography and fluorescence spectroscopy to characterize the short (2, 3 and 4-mer) RNA products made by RNAP during repeated rounds of abortive cycling before the enzyme progresses to forming full-length RNA transcripts. These combinatorial approaches have allowed us to determine the timing of loading of duplex DNA into the RNAP active site cleft and the kinetic mechanism of large-scale RNAP conformation changes leading to initial RNA synthesis. These research activities have successfully developed new methods to characterize transient intermediates in solution and to begin to develop a detailed structural-mechanistic picture of transcription initiation.

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A Magnetic Tweezers Study of a RNA-Dependant RNA Polymerase

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RNA-dependent RNA Polymerases (RdRPs) are involved in the transcription and the replication of RNA genome of many viruses. The RdRP of the bacteriophage Φ6 (P2) is located inside the viral capsid where it transcribes double-stranded RNA to yield single-stranded RNA that infects the host cytosol.

Here, we have studied the transcription activity of the P2 RdRP using magnetic tweezers. To do so, we tether a double-stranded RNA molecule between a glass surface and a magnetic bead. P2 then initiates at the free 3' extremity of one of the strands constituting the duplex and in the process of transcription, dehybridizes it from the other. By observing the ensuing conversion of the tether from a double-stranded RNA to a single-stranded RNA under a constant applied force, we detect P2 transcription dynamics. In this manner, we directly measure the polymerization rate and the distribution of the dwell times. These measurements suggest that P2 has the ability to backtrack over several nucleotides along the template.

Analysis of force dependence of the polymerization rate and pause density, as well as of the sequence-dependence of the polymerization rate indicates that the transcription activity of P2 RdRP is affected by the hybridization of the template and product strands. Though P2 is fully capable of unwinding and transcribing dsRNA in vitro unaided by other enzymes, we observe that the transcription velocity increases with increasing force, reaching a maximum at which the pause density is minimized. This behavior can be explained from the inability of the polymerase to backtrack at high forces where rehybridization is energetically unfavorable, diminishing the total time spent in pauses. The resulting relatively inefficient transcription process suggests that additional proteins may interact with the displaced strand inside the viral capsid to enhance overall transcription efficiency.

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E. Coli RNA Polymerase: A Molecular DNA Opening Machine

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Using ''burst'' kinetic methods to obtain near-homogeneous populations of transient intermediates for fast footprinting, together with solute probing experiments, we have characterized the series of large-scale conformational changes in *E. coli* RNA Polymerase (RNAP) and λP_R promoter DNA that occur in ''isomerization'' of the initial recognition complex to form the stable open complex (RP_o). We find that RNAP is a molecular isomerization machine that uses wrapping of upstream DNA to place the start-site region of the downstream duplex in the cleft (shown by HO protection and FRET in the closed intermediate I_1).[1] Then, in the rate determining step, RNAP opens the entire 13 bp initiation bubble (as judged by permanganate reactive thymines) and places the start site base of the template strand in the active site to form a transient open intermediate (I_2).[2] After this opening/ untwisting step, RNAP stabilizes the open DNA conformation by step-wise assembly of an encircling downstream jaw/ clamp, together with repositioning of the nontemplate strand in the cleft to form the more stable open intermediate I_3 and convert it to the stable open complex RP_o at λP_R.[2,3]

To test these conclusions, we are investigating deletions of the downstream ''jaw'' of RNAP [B'1149-1190], upstream and/ or downstream DNA, and/ or region 1.1 of σ^{70} subunit of RNAP [1-98] on the kinetics of formation and lifetime of open complexes. Remarkably, many of these deletions have large effects on the rate-determining, DNA-opening step, suggesting a synergy between upstream, downstream and in-cleft interactions in this sophisticated molecular machine.

1. Davis et al. '07; Drennan, Saecker *et al*., in preparation
2. Gries et al. '10
3. Kontur, Gries *et al*.'06, '08, '10

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